CURCUMINOID AND TOXICITY LEVELS OF ETHANOL EXTRACT OF JAVANESE GINGER (*CURCUMA XANTHORRHIZA*) ON BRINE SHRIMP (*ARTEMIA SALINA*) LARVAE AND ZEBRAFISH (*DANIO RERIO*) EMBRYOS

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ABSTRACT

Objective: This study was aimed at determining the levels of curcuminoids and analyzing the toxicity of ethanol extracts of Javanese ginger.

Methods: Curcuminoid levels were determined using high-performance liquid chromatography, while the toxicity tests were done on larva of brine shrimp (*Artemia salina*) by using a brine shrimp lethality test (BSLT) method and embryos of zebrafish (*Danio rerio*) using a zebrafish embryo acute toxicity (ZFET) method.

Results: The level curcuminoid of ethanol extracts was 10.5% dry wt., consisting of curcumin at the highest percentage (68.06%) followed by desmethoxycurcumin (24.6%) and bisdemethoxycurcumin (1.41%). In BSLT method, the lethal concentration 50% values (LC_{50}) value of our ethanol extract was 238 ppm, whereas in ZFET method, the LC_{50} value at 96 hours after fertilization was 80 ppm. The ethanol extract of ginger caused major malformations of the pericardial edema of zebrafish embryos at a concentration of 100 ppm.

Conclusion: The ethanol extract of *Curcuma xanthorrhiza* from Bogor contained curcuminoids consisting of curcumin, desmethoxycurcumin, and bisdemethoxycurcumin, with acute toxicity, caused major malformations on the pericardial edema in zebrafish embryos.

Keywords: *Curcuma xanthorrhiza*, Curcuminoid, Toxicity, Zebrafish embryo acute toxicity.

INTRODUCTION

Curcuma xanthorrhiza, known as temulawak, is a medicinal plant which grows in tropical areas like Indonesia. This plant is commonly grown in home yards. It has been known to have many benefits, particularly health benefits. The rhizome is the part of the plant which is usually used as a medicine. The rhizome of Javanese ginger is believed to have antimicrobial [1], anti-inflammatory [2], antioxidative [3], and anti-nociceptive properties [4]. Furthermore, a study by Cheah *et al.* showed that Javanese ginger had a potential as a growth inhibitor of human breast cancer [5].

These various health benefits of Javanese ginger are attributed to its main active components, namely curcuminoid, and essential oils. Curcumin and demetoxycurcumin were reported as the active components in *C. xanthorrhiza* [6]. The essential oils of Javanese ginger contained sesquiterpenoid compounds (β-curcumena, ar-curcumena), xantorizol, and a small amount of camphor [7]. An anti-inflammatory property of ethanol extract of Javanese ginger was reported by Itokawa *et al*. [8].

A preliminary toxicity test of a substance can be done using a brine shrimp lethality test (BSLT). This toxicity test has been proven to have a correlation with toxicity levels. As an indicator, shrimp larva is very sensitive to the toxic material so that the toxicity of material to shrimp larva can be used as an initial indication of the material's pharmacological effect. The BSLT method has some advantages including the fact that shrimp larva is sensitive to test materials, is easy to grow, and have a short life cycle. In addition, this method is easily performed, fast, and relatively inexpensive. However, there are also some disadvantages of this method. Results of the observation do not show the cause of death or type of abnormality of the test animal.

Therefore, a further test to assess the effects of active compounds on the growth of the test organism, by using a zebrafish embryo acute toxicity (ZFET) test, needs to be done.

Zebrafish (*Danio rerio*) is an animal originating from Myanmar, Srilanka, and India. Today, this fish is used in many kinds of tests, including toxicity testing. As a test animal, the zebrafish has several advantages, including the fact that this fish is small in size (about 2.5-4 cm), does not need plenty rearing space, has similar morphological and physiological structures to mammals, has external fertilization, and short life cycle. The embryo of this fish is transparent making direct observation *in vivo* on organ and tissue development possible [9]. In addition, the permeable embryo of zebrafish allows the distribution of active substances into the embryo [10] so that it can be used in a toxicity test. Therefore, toxicity types that can be assessed using the zebrafish bioassay including reproductive toxicity, acute toxicity, neurotoxicity, cardiotoxicity, optical toxicity, endocrine disorders, neurobehavioral toxicity, and vascular toxicity. This fish can also be used in anticancer testing [11].

This study was aimed at determining the level of curcuminoids and evaluating the toxicity of ethanol extracts of *C. xanthorrhiza* using the BSLT and the ZFET bioassays.

METHODS

Plant determination
C. xanthorrhiza was collected from the Conservation and Cultivation Unit, Biopharmaca Research Center, Bogor, Indonesia, in March 2014. The authenticity of the plant was confirmed by the Botany Division (Herbarium Bogoriense) of the Research Center for Biology Indonesian Institute of Sciences, Cibinong, Bogor, West Java, Indonesia.

Sample preparation

Javanese ginger rhizomes were washed in running water, surface dried, and chopped into small pieces. The rhizomes were then dried in an oven at 40°C for 24 hrs. Dried rhizomes were ground to obtain Javanese ginger powder.

Javanese ginger extraction

Preparation of Javanese ginger extraction was adopted from Srijanto and Syahbirin [12]. The extraction was done by macerating in 80% (v/v) ethanol as solvent. The ratio of Javanese ginger and solvent was 1:6. As much as 100 g of Javanese ginger powder was soaked in 80% ethanol in a macerator for 3 hrs at 40-45°C. The ginger bath was filtered using a filter paper to separate the filtrate from its macerate. The resulting filtrate was evaporated using a rotavapor (vacuum evaporator) to obtain a concentrated extract to be used in the next stages.

Determination of curcuminoid level using high-performance liquid chromatography (HPLC)

Curcuminoid level was measured using adapted method from Jayaprakasha *et al.* [13]. A 50 mg aliquot of Javanese ginger extract was dissolved in 50 mL methanol. The solution was diluted 50 times and filtered by using a Millipore 0.45 μm filter paper before it was put into HPLC vials. Curcuminoid standard was prepared at a concentration of 0.5 μg/mL. A C_{18} reverse-phase column with a visible-ultraviolet detector and 20 μL injection volume was used. Elution was done with a flow rate of 1.0 mL/minute. Methanol (A), acetic acid 2% (v/v) (B), and acetonitrile (C) were used as the eluent mix. A linear gradient elution was done with a constant A concentration (5%) and changing concentrations of C in B from 45 to 65% at zero time to 15 minutes to 65 to 45% at 15-20 minutes. The curcuminoid level was measured at wavelength of 425 nm.

Toxicity test of extract using *Artemia salina* larva

Preparation of toxicity test of extract using *A. salina* larva was adopted from Meyer *et al.* [14]. Eggs of *A. salina* were placed in a container filled in with filtered and aerated sea water. The eggs were kept illumination for 48 hrs so that the eggs hatched perfectly. The ethanol extract was dissolved in seawater in a concentration of 2000 ppm, and this solution was made the mother solution. This solution was then diluted to the concentrations of 100, 200, 300, 400, and 500 ppm. If the mixture was not clear, 100 μL dimethyl sulfoxide (DMSO) was added. Seawater, 10 shrimp larvae, and the Javanese ginger extract were put into each vial. Testing was done using three replicates. Vials were covered with aluminum foil sheet and incubated for 24 hrs. Lethal concentration 50% values (LC_{50}) were determined using a correlational curve between log^{10} extract concentration (x-axis) and probit value (y-axis).

Toxicity test of extract using zebrafish embryo

The husbandry and all procedures related with this study were approved by the Ethic Committee of Animal Use and Care, Faculty of Veterinary Medicine, Bogor Agricultural University, under license number 015/KEH/SKE/XI/2014. The toxicity test of extract using zebrafish embryo was adopted from OECD Guidelines [15]. Ethanol extracts of Javanese ginger were dissolved in filtered well water at a concentration of 2000 ppm, and this solution was made the stock solution. If the mixture was not dissolved well, DMSO was added. This stock solution was then diluted to the concentrations of 50, 100, 200, 300, 400, and 500 ppm. The toxicity test was done in 24 well plates. Each plate at each test concentration contained 20 zebrafish eggs (one embryo per well) and using 4 internal control plates, 1 negative control plate, and 1 solvent (DMSO) control plate. Each control plate contained 24 embryos (1 embryo per well). Plates containing zebrafish embryos were placed at room temperature (±26°C). The observation was carried out every 24 hrs until 96 hrs (4 days) using inverted microscopes (Olympus) equipped with a computer and camera. The observation was done on living or dead, normal or abnormal, hatched or unhatched (48 hrs only), and teratogenic abnormality. Types of abnormalities were observed include in body axis, head, tail, blood circulation, eyes, heart, pigmentation, somite, and yolk sac. LC_{50} were determined using

a correlational curve between log^{10} extract concentration (x-axis) and probit value (y-axis).

RESULTS AND DISCUSSION

Extract yield

The concentrated ethanol extract of Javanese ginger was a yellowish-brown paste. The yield of it was 17.77% on dry matter basis.

Curcuminoid analysis with HPLC

Curcuminoids are one of the secondary metabolites found in the largest amount in Javanese ginger [6]. Curcuminoids are a group of phenylpropanoid compounds consisting of curcumin, desmethoxycurcumin, and bisdemethoxycurcumin [16]. In this study, the curcuminoid content in ethanol extract of Javanese ginger was determined using HPLC. The standard curcuminoid used consisted of bisdemethoxycurcumin, desmethoxycurcumin, and curcumin. The HPLC chromatogram showed that the peak of standard curcuminoid was obtained at retention time of 7.847 minutes (bisdemethoxycurcumin), 8.463 minutes (desmethoxycurcumin), and 9.110 minutes (curcumin). The peaks in ethanol extract of Javanese ginger were found to be similar to those of the standards (Fig. 1). It was revealed that in ethanol extract of Javanese ginger, curcumin had the highest peak (68.06%) followed by desmethoxycurcumin (24.60%) and bisdemethoxycurcumin (1.41%).

These findings suggested that curcumin and desmethoxycurcumin were the dominant curcuminoids. This was in line with the findings of by Mangunwardoyo *et al.* [17] who reported that curcumin and desmethoxycurcumin were the dominant curcuminoids in Javanese ginger. In this study, the total content of curcuminoids was 10.5% dry wt.

Shrimp larva and zebrafish embryo toxicity tests

The BSLT can be used to predict the toxicity level of active components through the LC_{50} value which is the concentration of a substance needed to kill 50% of a population. LC_{50} values at less than 1000 ppm indicate that the tested extract is toxic. Meanwhile, in the ZFET, the extract is considered toxic if growth inhibition or organ abnormalities in zebrafish embryos are observed.

LC values were determined by using a correlational curve between log^{10} extract concentration (x-axis) and probit value (y-axis). In this study, the ethanol extract tested on shrimp larvae resulted in an LC_{50} value of 238.23 ppm (Table 1). This was not too different from the one (210.30 ppm) found by Nurcholis *et al.* [18].

It is shown in Table 1 that in the ZFET test, LC_{50} values decreased with increased time. LC_{50} value at 96 hpf was lower than that at 48 hpf indicating that the material was more toxic as the test animal was exposed for a longer time. This might be caused by the fact that the body immunity of zebrafish decreased with longer exposure, while the extract concentration remained constant.

Overall, LC_{50} values obtained in the BSLT were higher than those in the ZFET. In the BSLT, the extract was added when the *A. salina* had already formed larvae while in the ZFET, extract addition was done when zebrafish was in the form of sensitive embryos. In addition, the BSLT was only done over 24 hrs, while the observations in the ZFET was done over 96 hrs, allowing a longer time for the extract to accumulate in the embryos. The toxicity of curcumin on normal cell was reported

Table 1: LC_{50} values of ethanol extract tested on shrimp larva (BLST) and ZFET

Test type	LC_{50} (ppm)
BSLT	238.23
ZFET 48 hpf	180.52
ZFET 96 hpf	79.55

hpf: Hours post fertilization, BSLT: Brine shrimp lethality test, ZFET: Zebrafish embryo acute toxicity, LC_{50}: Lethal concentration 50% values

previously using fibroblastic L929 cells showed the percentage viability of cells was 55.52% in concentration 50 µg/mL (ppm) during 24 hrs exposure time [19]. This report support different cell or stage of development or exposure time of curcumin influenced of toxicity level. The LC_{50} values obtained in this study were less than 1000 ppm, indicating that the extract used in both tests was toxic.

Teratogenic effects of ethanol extract on zebrafish embryos
Embryos of zebrafish were microscopically assessed for the effects of the extract on the morphology of the embryos. An inhibitory effect resulted from an extract on embryo development causing growth abnormalities is known as a teratogenic effect. The teratogenic effects of ethanol extract on organs and tissues of zebrafish embryos are listed in Table 2. Malformation is considered to be major if it is found in ≥50% of examinations, and minor if <50% of examinations.

Results of the observation showed that our ethanol extract gave some teratogenic effects on the development of zebrafish embryos. It is shown in Tabel 1 that a major malformation in embryos treated with ethanol extract was identified for the heart malformation, i.e the pricardial edema.

Table 2: Teratogenic effects of ethanol extract exposure in zebrafish embryos

Body part/organ	Abnormality	
	Σ^a	(%)[b]
Body axis	7	35
Brain	0	0
Tail	5	25
Blood circulation	0	0
Eyes	0	0
Heart	10	50*
Jaws	0	0
Otolith	0	0
Pigmentation	3	15
Somite	9	45
Yolk sac	5	25

[a]Number of embryos with teratogenic effects in all concentration and treatment times. [b]Number of embryos with teratogenic effects/number of embryos with abnormalities in all concentration and treatment times. *Major malformation (≥50%). One embryo may suffer from more than 1 malformation

Minor malformation was identified in body axis, tail, pigmentation, somite, and yolk sac of embryos treated ethanol extract. Embryogenesis in zebrafish is similar to that in higher level vertebrates, including humans, making the zebrafish an ideal model animal for studies in the embryo development process embrio [20,21]. In the negative control, embryo development in eggs was observed in 24 hpf (Fig. 2a). At 48 hpf, embryos developed normally into larvae with perfectly formed organs and a straight body axis (Fig. 2b). Larva of zebrafish is considered normal if it has a straight body axis [22]. At 48 hpf, only 50% embryos treated with 50 ppm concentration hatched. This finding shows that at 50 ppm concentration, our ethanol extract had inhibitory effects on embryo development. The dominant malformation found in 50 ppm treatment was abnormality in the tail (Fig. 2c).

The active compounds of the extract entering pericardial sac could irritate the cells making the heart appear swollen. Exposure with 100 ppm ethanol extract made 70% of the embryos unhatchable at 48 hpf. Compared to those in the control group, larvae in the treatment groups had dominant malformations including a crooked body axis (Fig. 2d, 72 hpf), pericardial edema (Fig. 2e, 96 hpf), and yolk sac edema (Fig. 2f, 72 hpf). The yolk sac functions as a nutrition supply for the embryo. Yolk sac edema is an indication of impaired nutrient adsorption in embryo. This impairment might be caused by malformation in other organs assisting nutrient distribution. This impairment might also lead to the inhibition of nutrient adsorption, undernourished embryos, and the death of embryos. Treatment at a concentration 200 ppm extract (Fig. 2g) made the embryos fail to hatch although some embryo development in the eggs was observed. Embryo failures to develop beyond the chorion could be caused by the failure of the somite to develop. Somite is a tissue which will further differentiate into the backbone and skeletal muscles. Therefore, abnormality in somite development might result in malformation of the body axis and inhibit the hatching process [23]. Treatments at concentrations of 400 and 600 ppm (Fig. 2h) resulted in early death of embryos in the chorion.

Wu *et al.* [24] reported that the teratogenic effects of curcumin treatment on zebrafish embryos included bent or hook-like tails, spinal column curving, edema in pericardial sac, retarded yolk sac resorption, and shorter body length. The teratogenic effects we found in zebrafish embryos exposed to our ethanol extract were similar. This indicates that
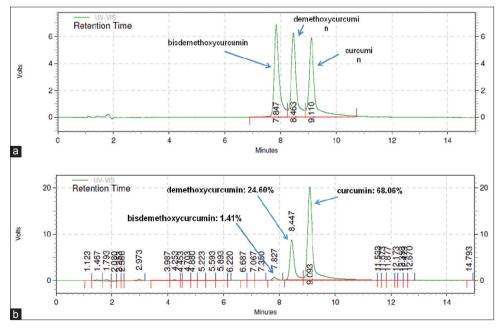

Fig. 1: High-performance liquid chromatography chromatograms of (a) standard curcuminoids and (b) ethanol extract of curcuminoids (*Curcuma xanthorrhiza*)
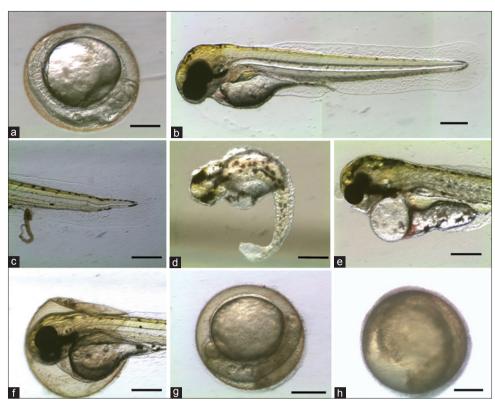
Fig. 2: Morphology of normal zebrafish embryo in control group and some abnormalities in ethanol treatment groups: Normal embryo K-24 hpf (a), K-48 hpf (b), tail malformation (72 hpf, 50 ppm) (c), body axis malformation (72 hpf, 100 ppm) (d), pericardial edema (96 hpf, 100 ppm) (e), yolk sac edema (72 hpf, 100 ppm) (f), 48 hpf, 200 ppm treatment (g), 24 hpf 400 and 600 ppm treatment (h). K: Negative control, hpf: Hour(s) post fertilization (bar 300 μm)

curcumin was the active compound in ethanol extract. This result was in line with the results of HPLC analysis which showed that compared to the other two curcuminoids, cucurmin had the highest content.

CONCLUSION

Based the results of HPLC, the ethanol extract of *C. xanthorrhiza* from Bogor contained curcuminoids consisting of curcumin at the highest percentage (68.06%) followed by desmethoxycurcumin (24.6%) and bisdemethoxycurcumin (1.41%). In the BSLT method, the LC_{50} value of our ethanol extract was 238 ppm, whereas for the ZFET method we observed LC_{50} values of 96 hpf at 80 ppm. The ethanol extract of *C. xanthorrhiza* at concentration of 100 ppm caused major malformations of the heart of zebrafish embryos.

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